Atty. Docket No.: 4231/2055K PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Choong-Chin Liew	Examiner:	Juliet Switzer
Serial No.:	10/813,097		
Filed:	March 30, 2004	Group Art Unit:	1634
Titled:	Method for the Detection of Chagas Disease Related Gene Transcripts in Blood		
		Conf. No.:	5239

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF Hongwei Zhang UNDER 37 C.F.R. §1.132

Sir:

I, **Hongwei Zhang**, Ph.D., hereby declare that:

1. I received a Ph.D. degree from the Institute of Medical Science at the University of Toronto in 2002, and a Master of Science degree from the Department of Immunology at the University of Toronto in 1995. In addition I received my Medical Degree from the University of Medical Sciences in Changchun China in 1989 and practiced as a staff physician for 4 years in Beijing prior to commencing my post graduate studies. I currently hold the position of Director of Biomarker Development at GeneNews Corporation (formerly ChondroGene Ltd., the Assignee of the application).

I am a trained molecular biologist experienced in developing methods to identify biomarkers which are indicative of a disease or condition, and in developing methods of using these biomarkers and products thereof as applied in the area of Chagas disease, amongst other conditions.

List of Publications:

K.W. Marshall, M.D., PhD., F.R.C.S., **H. Zhang, M.D., PhD.**, T.D. Yager Ph.D., N. Nossova M.D., Ph.D., A. Dempsey PhD., R. Zheng M.D., M. Han M.D. Ph.D., H.Tang M.Sc., S. Chao M.A.Sc, and C.C. Liew PhD. "Blood-based biomarkers for detecting mild osteoarthritis in the human knee" *OsteoArthritis and Cartilage (2005)* 861-871.

Zhang H, Marshall KW, Tang H, Hwang DM, Lee M, Liew CC. Profiling genes expressed in human fetal cartilage using 13,155 expressed sequence tags. Osteoarthritis Cartilage 2003;11:309-19.

Hongwei Zhang, C.C.Liew, K.Wayne Marshall. Microarray Analysis Reveals the Involvement of Beta-2 Microglobulin (B2M) in Human Osteoarthritis. Osteoarthritis and Cartilage 2002;10:950-60.

Doherty PJ, **Zhang H,** Manolopoulos V, Trogadis J, Tremblay L, Marshall KW. Adhesion of transplanted chondrocytes onto cartilage in vitro and in vivo. J Rheumatol 2000;27:1725-312.

Zhao YX, Lajoie G, **Zhang H,** Chiu B, Payne U, Inman RD. Tumor necrosis factor receptor p55-deficient mice respond to acute Yersinia enterocolitica infection with less apoptosis and more effective host resistance. Infect Immun 2000;68:1243-513.

Vaselios Manolopoulos, K. Wayne Marshall, **Hongwei Zhang,** Judy Trogadis, Louise Trembly and Paul J. Doherty. Factors affecting the efficacy of bovine chondrocyte transplantation in vitro. Osteoarthritis and Cartilage 1999;7:453-460.

Yi-Xue Zhao, **Hongwei Zhang,** Basil Chiu, Usulra Payne, Robert D. Inman. Tumor necrosis factor receptor P55 controls the severity of arthritis in experimental *Yersinia Enterocolitica* infection. Arthritis & Rheumatism 1999;42:1662-1672.

Paul J. Doherty, **Hongwei Zhang,** Louise Trembley, Vaselios Manolopoulos and K. Wayne Marshall. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes *in vitro*. Osteoarthritis and Cartilage 1998;6:153-160.

Hongwei Zhang, Donna Phang, Ronald M. Laxer, Earl D. Silverman, Sueihua Pan, and Paul J. Doherty. Evolution of the T cell receptor beta repertoire from synovial fluid T cells of patient with juvenile onset rheumatoid arthritis. J. Rheumatol. 1997;24:1396-402.

Petro Lastres, Anihoa Letamendia, **Hongwei Zhang,** Carlos Rius, Nuria Almendro, UIIa RAab, Louis A. Lopez, Carmen Langa, Angels Fabra, Michelle Letarte and Carmelo Bernabeu. Endoglin modulates cellular responses to TGF-beta 1. J. Cell Biol. 1996;133:1109-1121.

Hongwei Zhang, Andrew R.E. Shaw, Allan Mak, and Michelle Letarte. Endoglin is a component of the Transforming Growth Factor (TGF)-beta receptor complex of human pre-B leukemic cells. J. Immunol. 1996, 156:565-573.

2. I have read the non-final Office Action mailed June 7, 2007 in the above-referenced patent application.

In providing grounds for rejection of claims under 35 U.S.C. § 112(1), the Examiner asserts at page 9 of the Office Action: "*It is not known under what circumstances the result observed in the instantly examined control and test populations would be repeatable, as the results have not been validated.*"

3. As a scientist skilled in the area of molecular biomarker identification, I submit that post-filing validation experiments performed by the Assignee of the present application using both quantitative RT-PCR (QRT-PCR), an alternate technology relative to microarray analysis employed in the experiments disclosed at Example 28 and Table 3Z of the specification, as well as an independent cohort of control and disease subjects relative to those employed in the experiments disclosed at Example 28 and Table 3Z of the specification, have shown that RNA encoded by the gene CDC14A is present at statistically higher levels in blood of subjects having Chagas disease relative to healthy control subjects.

Levels of CDC14A expression in blood are statistically higher in Chagas disease patients versus healthy control subjects – validation of CDC14A as biomarker of Chagas disease in blood via an alternate technology (quantitative RT-PCR) using an independent cohort

Attached as Exhibit "A" to this Declaration are results and materials and methods of post-filing experiments performed by the Assignee of the present application in which levels of CDC14A-encoded RNA in blood were found to be statistically higher in subjects having Chagas disease relative to healthy control subjects, as determined via QRT-PCR, an alternate technology relative to the microarray analysis employed in the experiments disclosed at Example 28 and Table 3Z of the specification, and with an independent cohort of control and disease subjects relative to that described at Example 28 and Table 3Z of the specification. As shown in Tables 1 and 2 of Exhibit "A", the average level of CDC14A-encoded RNA in blood samples from 5 Chagas disease patients, as determined via $\Delta\Delta$Ct values obtained from QRT-PCR analysis, was found to be significantly higher (1.6-fold)

relative to that of 4 healthy control subjects tested, with the difference in expression levels being statistically significant (p=0.033).

In view of the above, I submit that the specification enables one of skill in the art to practice the claimed methods.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Hongwei Zhang, Ph.D. _____

Date December 7, 2007

EXHIBIT "A"

TABLE 1. Quantitative RT-PCR analysis of CDC14A-encoded RNA levels in blood of Chagas disease patients versus healthy control subjects.

Experimental group	Sample ID	Relative fold-change
Healthy control	EC04	1.39
	EC05	1.06
	EC09	0.77
	EC10	0.87
Chagas disease	EC03	1.34
	EC06	2.25
	EC07	1.19
	EC08	1.78
	EC164	1.80

TABLE 2. Analysis of QRT-PCR data of Table 1, above, for Chagas disease detection.

Relative average level of CDC14A expression (relative fold-change)	**healthy control subjects**	1.03
	Chagas disease patients	1.67
Average fold-change CDC14A expression (Chagas disease patients/control subjects)		1.6
***p*-value**		0.033

Materials and Methods:

Blood RNA Isolation: Samples were obtained from 5 patients diagnosed with Chagas disease, and from 4 healthy control subjects. All participants provided written informed consent. Approximately 10ml of blood was collected from each participant, using a Vacutainer™ tube (Becton Dickinson, Franklin Lakes, NJ). Red blood cells were ruptured with hypotonic lysis buffer (1.6 mM EDTA, 10 mM KHCO3, 153 mM NH4Cl, pH 7.4), followed by collection of white blood cells by centrifugation. White blood cell total RNA was extracted with Trizol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. The quality of RNA samples was assessed on an Agilent Bioanalyzer 2100 using RNA 6000 Nano Chips (Agilent Technologies, Palo Alto, CA), and the quantities of RNA were measured by UV spectrophotometry (Beckman-Coulter DU640).

Real-time QRT-PCR: Real-time QRT-PCR was used to measure levels of CDC14A-encoded RNA in blood samples from Chagas disease patients and healthy control subjects. First strand cDNA was synthesized from 1μg total RNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a volume of 100μl, consisting

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of 10µl 10x RT buffer, 4µl 100mM dNTP mix, 10µl 10x RT random primers and 5µl Multi-scribe reverse transcriptase (50U/µl). Real-time PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in a reaction volume of 25µl consisting of 12.5µl 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5µl of 5pmol primer mix (sense primer, GCTTCGAGCACTATGACCTCTT; anti-sense primer, TCCAAGACCAGCTTTGCAGT; both primers are optimized for an annealing temperature of 56°C), and 2.5ng first strand cDNA. The PCR cycling protocol used is as follows: (1) 50°C, 2 min; (2) 95°C, 10 min; (3) 40 cycles of 95°C, 15 sec; 60°C, 1 min; and (4) determining the dissociation curve from 60°C to 95°C. The gene GAPDH was used as the housekeeping gene for normalization.

Fold-change/statistical analysis: A Welch's t-test was applied to the fold-change values to test for the statistical significance of the difference in RNA levels between the disease and healthy control groups. Fold change was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ was calculated by subtracting the mean ΔCt value of the control samples from the ΔCt of each sample for each gene. Statistical analysis was performed using SigmaStat v3.0 (SPSS Scientific, Chicago, IL).